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Mutant Macaque Factor IX T262A: A Tool for Hemophilia B Gene Therapy Studies in Macaques

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ABSTRACT

Introduction: Gene therapy is expected to be the next generation therapy for hemophilia, and a good animal model is required for hemophilia gene therapy preclinical studies.
Methods: Taking advantage of the human factor IX (FIX) specificity of monoclonal antibody 3A6, the epitope of which resides in the amino acid polypeptide segment including Ala 262 of human FIX, mutant macaque FIX with an amino acid substitution of Thr 262 to Ala (macaque FIX T262A) was generated and its reactivity to monoclonal antibody 3A6, biological activity and expression *in vivo* were studied.
Results: Enzyme-linked immunosorbent assays (ELISAs) and Western blot analyses showed that monoclonal antibody 3A6 bound to human FIX and macaque FIX T262A but not to wild-type macaque FIX. Recombinant macaque FIX T262A exhibited a comparable coagulation activity to wild-type macaque FIX and human FIX. High expression of macaque FIX T262A was achieved in mice by injection of AAV8 vectors carrying the macaque FIX T262A gene and reached levels of up to 31.5 μg/mL (1050% of the normal human FIX concentration). Macaque FIX T262A expressed in the liver of mice was as biologically active as that expressed *in vitro*. In addition, the macaque FIX T262A concentrations determined by a 3A6-based ELISA were not influenced by the presence of normal macaque plasma.
Conclusions: The results of the present study suggest that macaque FIX T262A may be processed appropriately *in vivo* and that the macaque FIX T262A concentration in the macaque circulation can be quantified precisely by a monoclonal antibody 3A6-based ELISA.

A wide variety of disorders are caused by genetic abnormalities, thereby giving rise to enthusiasm for gene therapy as the next generation therapeutics for many diseases [1–4]. Indeed, many gene therapy clinical trials have been conducted, and some have achieved great successes [5–7]. However, others have been unsuccessful. Furthermore, unpredicted adverse effects occurred in some trials [8,9]. To establish gene therapy technologies, good animal models are required. Advances in developmental biotechnology have allowed us to create a variety of mouse disease models, transgenic mice and gene-targeted mice. However, there are significant species differences between humans and mice, thus making it difficult under certain circumstances to extrapolate data obtained in mice to human patients [10]. Factor IX (FIX)-deficient mice (hemophilia B mice) and natural hemophilia B dogs have been used to study gene therapy approaches for the treatment of hemophilia B [3,11–13]. However, better animal models may be required because of the limited success in human trials [9,10]. Primates are used successfully as models in disease applications, but there are presently no hemophilic primates available for gene therapy studies. If one can distinguish human molecules from primate molecules *in vivo*, non-human primates may be used for hemophilia gene therapy preclinical studies [11,14,15], despite the fact that this genetic abnormality is not indigenous to these species.

Rhesus macaques have been proposed as a good primate model for hemophilia B gene therapy studies because of the amino acid sequence similarity between human FIX and macaque FIX and the low immunogenicity of human FIX in rhesus macaques [16]. However,

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rhesus macaques still developed antibodies against human FIX upon receiving adenoviral vectors carrying the human FIX gene despite the high amino acid sequence homology [17], thus making it difficult to study the long-term expression of transgene-derived FIX. We previously reported that a human FIX-specific monoclonal antibody, 3A6, which can distinguish human FIX from cynomolgus macaque FIX in enzyme immunoassays and Western blot analyses, binds to the amino acid segment including the Ala residue at position 262 of the human FIX molecule [18]. Only one amino acid residue at position 262 in this segment differs between macaque FIX and human FIX [16,19]. Therefore, the Thr residue at position 262 of macaque FIX was mutated to Ala (macaque FIX T262A) to examine whether the human FIX-specific monoclonal antibody 3A6 could bind to the mutant macaque FIX in the present study. Here, we show that macaque FIX T262A binds to monoclonal antibody 3A6 and is as active as wild-type macaque FIX and human FIX, that macaque FIX T262A can be efficiently expressed *in vivo* in mice by injection of AAV8 vectors carrying the mutant macaque FIX gene, and that quantification of macaque FIX T262A by a 3A6-based ELISA was not influenced by the presence of macaque plasma. These findings raise the possibility of a potential advantage of mutant macaque FIX T262A over human FIX to study the long-term expression of a FIX transgene in macaques.

Materials and Methods

Cloning of macaque FIX cDNA

Total RNA was isolated from the cynomolgus macaque liver using an RNeasy Mini Kit (Qiagen Inc., Valencia, CA). The isolated RNA was subjected to RT-PCR to amplify the macaque FIX cDNA based on the nucleotide sequence of a macaque FIX cDNA [19] using *pfu* Turbo DNA polymerase (Stratagene, La Jolla, CA) and a primer pair (5′-AGG TTA TGC AGC GCG TGA AC-3′/5′-CCA TCT TTC ATT AAG TGA GCT TTG-3′). The DNA fragment of the macaque FIX cDNA was cloned into the plasmid vector pCR Blunt II using a Zero Blunt TOPO PCR Cloning Kit (Invitrogen Co., Carlsbad, CA) and sequenced. The macaque FIX cDNA was subjected to site-directed mutagenesis to create the cDNA expressing macaque FIX T262A using a QuikChange Site-directed Mutagenesis Kit (Stratagene) and a primer pair (5′-CCT CAT CAC AAC TAC AAT GCA GCT ATT AAT AAG TAC AAC CAT G-3′/5′-CAT GGT TGT ACT TAT TAA TAG CTG CAT TGT AG T TGT GAT GAG G-3′).

Construction of FIX minigenes

A human FIX cDNA was a generous gift from Dr. George G. Brownlee (Chemical Pathology Unit, University of Oxford, Oxford, UK). The DNA fragment spanning part of exon 1, intron 1 and part of exon 2 of the human FIX gene was amplified from the human gene by PCR and sequenced. After removing part of intron 1 of the FIX gene by Pvu II treatment, the modified DNA fragment spanning parts of exon 1, intron 1 and exon 2 of the human FIX gene was excised with Bcl I and cloned into the Bcl I recognition sequence of the human FIX cDNA in the appropriate orientation to create a FIX minigene that was shown to express human FIX more efficiently than the FIX cDNA [10]. Similarly, the modified DNA fragment spanning parts of exon 1, intron 1 and exon 2 of the human FIX gene was cloned into the Bcl I recognition sequences of the macaque FIX cDNA to create a chimeric macaque FIX minigene.

Expression of macaque FIX in vitro

The human FIX minigene, macaque FIX minigene and mutant macaque FIX T262A minigene were cloned into plasmid p1.1c (Avigen Inc., Alameda, CA) in the appropriate orientation to create the plasmids p1.1c-hFIX, p1.1c-macFIX and p1.1c-macFIXT262A expressing human FIX, wild-type macaque FIX, and mutant macaque FIX T262A, respectively, under the control of the CMV promoter. Human embryo kidney (HEK) 293 cells were transfected with p1.1c-hFIX, p1.1c-macFIX and p1.1c-macFIXT262A in the presence of vitamin K (10 μg/mL), and the human FIX and macaque FIX expression levels in the conditioned media of the HEK 293 cells were analyzed by enzyme-linked immunosorbent immunoassays (ELISAs) and Western blot analyses. The FIX clotting activities in the conditioned media were determined by the APTT method using FIX-deficient human plasma (Dade Behring, Siemens Healthcare Diagnostics, Deerfield, IL).

Cloning of the hepatic control region (HCR) of the ApoE/C-I gene locus and the human α1 antitrypsin promoter (HAAT)

The 325-bp DNA fragment spanning the HCR region of the ApoE/C-I locus (nucleotides 1-325) [20–23] was amplified by PCR with a primer pair (sense primer, 5′-CAC TAG TCT GCA GGC TCA GAG GCA CAC-3′; antisense primer, 5′-GAA CCC GGA CCC TCT CAC ACT AC-3′) and cloned into the plasmid vector pCR Blunt II. The 297-bp DNA fragment spanning the HAAT promoter (nucleotides -270 to +27) was amplified by PCR and cloned as described previously [24].

AAV vector production

Plasmid vector p1.1c was excised with Spe I and Eco RI to remove the DNA fragments spanning the CMV promoter and the growth factor gene intron 1, and the DNA fragment of HCR was inserted into the same position. Subsequently, the DNA fragment of the HAAT promoter was cloned into the Eco RI site in the appropriate orientation to create the plasmid p1.1HCRHAAT. The macaque FIX T262A minigene was cloned on the 3′ side of the HAAT promoter of p1.1HCRHAAT in the appropriate orientation to create p1.1HCRHAAT-macFIXT262A. The DNA fragments spanning the promoter, LacZ gene and polyadenylation signal sequence of pAAV2-LacZ (Stratagene) were replaced with DNA fragments spanning the CMV promoter, macaque FIX T262A minigene and SV40 polyadenylation signal sequences of p1.1c-macFIXT262A to create the gene transfer vector pAAV2-CMV-macFIXT262A, in which these DNA fragments were flanked by ITR sequences of AAV serotype 2 (AAV2) as described previously [24,25]. Similarly, pAAV2-HCRHAAT-macFIXT262A was constructed by replacing the DNA fragment between the two ITRs of pAAV2-LacZ with the DNA fragment spanning the HCRHAAT, macaque FIX T262A minigene and polyadenylation signal sequences of p1.1HCRHAAT-macFIXT262A. The vector production system and HEK 293 cells were kindly supplied by Avigen Inc. The AAV vectors were packaged with the AAV8 capsid by pseudotyping. The chimeric packaging plasmid for AAV8 capsid pseudotyping was a generous gift from Dr. James M. Wilson (Division of Medical Genetics, Department of Medicine, University of Pennsylvania, Philadelphia, PA). For virus vector purification, virus particle-containing samples were treated with DNase (Benzonase; Merck Japan, Tokyo, Japan) and subjected to two rounds of cesium chloride-density gradient ultracentrifugation in HEPES-buffered saline (pH 7.4) in the presence of 25 mM EDTA at 21 °C as described [24,25]. Titration of the recombinant AAV vectors was carried out by quantitative PCR as described previously [24] with the primers 5′-GGT TGT TGG TGG AGA AGA TGC-3′ and 5′-GAT AGA GCC TCC ACA GAA TGC A-3′, and the probe 5′-FAM- GAT AGA GCC TCC ACA GAA TGC A-3′.

Animal experiments

Male C57BL/6 wild-type mice were purchased from SLC Inc. (Hamamatsu, Japan) and maintained under standard lighting conditions in a clean room. All surgical procedures were carried out in accordance with guidelines approved by the Institutional Animal Care and Concern Committee at Jichi Medical University. Before and after AAV vector injection, blood was drawn from the cervical vein plexus

of the mice and mixed with a 1/10 volume of 3.8% sodium citrate, before platelet-poor plasma was prepared by centrifugation. The AAV vectors were injected into the cervical vein plexus of the mice under anesthesia with isoflurane. Cyclophosphamide (100 μg/body/day; Sigma-Aldrich Japan, Tokyo, Japan) and tacrolimus (12.5 μg/body/day; Fujisawa Pharmaceuticals Co., Tokyo, Japan) were subcutaneously administered to the mice 5 times a week for 12 weeks after the vector injection as an immunosuppressant. Mouse plasma samples were subjected to an ELISA for human FIX to determine the plasma concentrations of macaque FIX T262A. Macaque plasma and macaque liver tissues were obtained from macaques under anesthesia according to the Institutional Animal Care and Concern Committee at Tsukuba Primate Research Center.

ELISA for human FIX

The ELISA that detects human FIX but not wild-type macaque FIX was performed as described previously [18]. Monoclonal antibody 3A6-coated microtiter plates (Maxisorp; Nalge Nunc, Rochester, NY) were blocked with 5% casein and then incubated with FIX-containing samples (conditioned media or mouse plasma samples) in phosphate-buffered saline (pH 7.4) containing 1% casein and 0.1% Triton X-100. Monoclonal antibody 3A6-bound human FIX or macaque FIX was detected with a horseradish peroxidase (HRP)-conjugated goat anti-human FIX polyclonal antibody (Affinity Biologicals Inc., Hamilton, Ontario, Canada). Purified human FIX was used as a standard. The ELISA that detects human FIX, wild-type macaque FIX and macaque FIX T262A was carried out using a sheep anti-human FIX polyclonal antibody (Cedarlane Laboratories Ltd., Hornby, Ontario, Canada) and an HRP-conjugated goat anti-human FIX polyclonal antibody (Affinity Biologicals Inc., Ancaster, Canada). Briefly, microtiter plates coated with the sheep anti-human FIX polyclonal antibody (1 μg/mL) were incubated with samples containing FIX. After washing, the microtiter plate-bound FIX was detected with the HRP-conjugated goat anti-human FIX polyclonal antibody.

Results

Binding of monoclonal antibody 3A6 to macaque FIX T262A

Monoclonal antibody 3A6 has been shown to be able to distinguish human FIX from wild-type macaque FIX [18]. Furthermore, it binds to wild-type FIX but not to mutant human FIX with an amino acid substitution of Ala to Thr (the amino acid residue at position 262 of macaque FIX is Thr), suggesting that the epitope for 3A6 resides in the segment including the Ala residue at position 262 of human FIX. Based on these data, we isolated a macaque FIX cDNA and expressed wild-type macaque FIX and mutant macaque FIX with an amino acid substitution of Thr to Ala at position 262 (macaque FIX T262A). Wild-type macaque FIX, mutant macaque FIX T262A and wild-type human FIX were expressed in HEK 293 cells and evaluated for their biological activities and reactivity with monoclonal antibody 3A6. The antigen concentrations of recombinant FIX were quantified by ELISAs using a sheep polyclonal antibody against human FIX and an HRP-conjugated goat polyclonal antibody against human FIX. The polyclonal antibody-based ELISA detected wild-type macaque FIX and mutant macaque FIX T262A, and all the FIX molecules had coagulation activities with similar specific activities (Table 1). Wild-type macaque FIX was not detected by the 3A6-based ELISA, whereas macaque FIX T262A and wild-type human FIX were quantified in a similar manner by this ELISA. The antigen concentrations of macaque FIX T262A determined by the polyclonal antibody-based ELISA were slightly higher than those by 3A6-based ELISA. This may be due to FIX fragments found in the supernatant of vector-transfected 293 cells.

The above data were confirmed by Western blot analyses (Fig. 1), which also showed that 3A6 bound to human FIX and macaque FIX T262A, but not to wild-type macaque FIX, while the polyclonal anti-FIX antibody bound to all the FIX molecules.

Table 1
Recombinant factor IX expression *in vitro*.

	Activity (U/mL)	Antigen (ng/mL)	
		Polyclonal Ab ELISA	3A6 ELISA
Wild-type human FIX	0.031	260	100
Wild-type macaque FIX	0.017	120	0
Macaque FIX T262A	0.024	200	101

Polyclonal Ab ELISA: the solid-phase (catching) antibody was a polyclonal anti-human FIX antibody.
3A6 ELISA: the solid-phase (catching) antibody was monoclonal anti-human FIX antibody 3A6.
The normal human plasma FIX concentration is 3 μg/mL by the 3A6 ELISA.

Expression of mutant macaque FIX T262A in vivo

AAV8 vectors carrying the macaque FIX T262A gene under the control of the CMV promoter (AAV8-CMV-macFIXT262A) or HCRHAAT promoter (AAV8-HCRHAAT-macFIXT262A) (Fig. 2) were injected into wild-type mice, and the expression of macaque FIX T262A was analyzed by the 3A6-based ELISA. Macaque FIX T262A was efficiently expressed in the mice using the AAV8 vectors, and high macaque FIX T262A expression was observed for more than 50 weeks (Fig. 3). In particular, the concentration of macaque FIX T262A in mouse plasma increased to supernormal levels (maximum: 14.4-31.5 μg/mL, 480-1050% of the normal plasma human FIX concentration) with AAV8-HCRHAAT-macFIXT262A at a dose of 5×10^{9} vector genome/g. Such high FIX transgene expression was also achieved with the AAV9 vector carrying the same promoter and the macaque FIX T262A gene (data not shown). The mouse plasma samples containing macaque FIX T262A at 5.4-14.4 μg/mL (180-480% of the normal plasma human FIX concentration) were diluted and subjected to the coagulation assay for FIX to study the biological activity of macaque FIX T262A expressed *in vivo*. After subtraction of the basal mouse FIX activity, the FIX activity of mouse plasma containing macaque FIXT 262A at the immunological concentration of 10.9 ± 3.9 μg/mL ($n=5$) was 4.7 ± 9.3 U/mL ($n=5$), indicating that macaque FIX T262A expressed in mice was biologically active. The plasma levels of macaque FIX T262A in AAV8-HCRHAAT-macFIXT262A-injected mice were approximately 100-fold higher than those in AAV8-CMV-macFIXT262A-injected mice, suggesting that the HCRHAAT promoter worked more efficiently than the CMV promoter *in vivo*.

Detection of macaque FIX T262A in the presence of macaque plasma

To confirm that the 3A6-based ELISA could distinguish macaque FIX T262A from wild-type macaque FIX, recombinant macaque FIX T262A-containing samples were subjected to the 3A6-based ELISA in

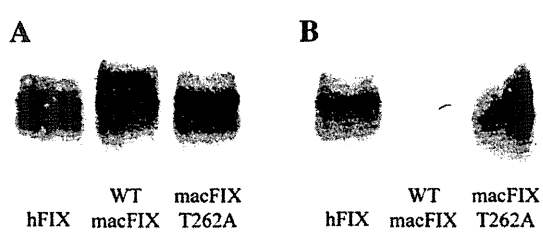

Fig. 1. Western blotting analyses of FIX molecules. Human FIX (hFIX), wild-type macaque FIX (WT macFIX) and macaque FIX T262A (macFIXT262A) expressed in the conditioned media of HEK 293 cells transfected with plasmid vectors carrying the corresponding FIX genes were analyzed by Western blotting with a polyclonal antibody against human FIX (A) and monoclonal antibody 3A6 (B).

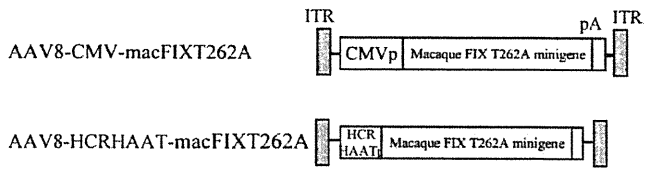

Fig. 2. Schematic representations of AAV8 vectors carrying the macaque FIX T262A gene. The AAV8 vectors carrying the macaque FIX T262A gene used in the present study are schematically illustrated. The promoter sequences, macaque FIX T262A gene and SV40 polyadenylation signal sequences are flanked by AAV2 ITRs.

the presence of increasing concentrations of macaque plasma to analyze the effect of wild-type macaque FIX in the plasma. As shown in Fig. 4, the presence of macaque plasma exhibited no inhibitory effects on the quantification of recombinant macaque FIX T262A expressed *in vivo* by the 3A6-based ELISA. These data confirm that the 3A6-based ELISA can be used to quantify macaque FIX T262A expressed in macaques.

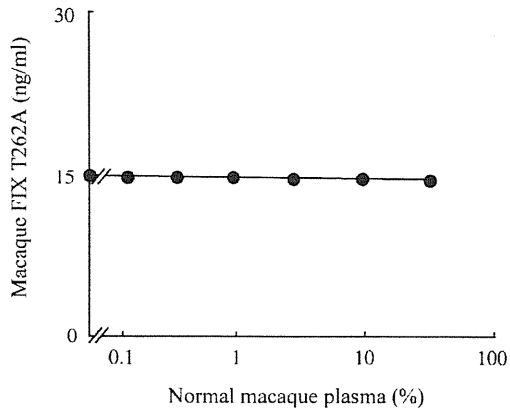

Fig. 4. Macaque plasma has no effect on the quantification of macaque FIX T262A by the 3A6-based ELISA. Mouse plasma containing macaque FIX T262A was diluted with buffer to adjust the macaque FIX T262A concentration to 30 ng/mL, mixed with equal amounts of buffer containing increasing concentrations of normal macaque plasma, and subjected to the 3A6-based ELISA for macaque FIX T262A quantification.

Discussion

To develop gene therapy technologies, good animal models are required. Hemophilia B mice (FIX-deficient mice) and natural hemophilia B dogs are available and have been used to study gene therapy approaches for hemophilia B. In a previous FIX gene transfer study with AAV2 vectors, a vector dose of 1.8×10^{12} vector genome/kg yielded plasma FIX levels of more than 1% in mice, whereas the same vector dose yielded circulating FIX levels of 0.2-0.4% in dogs. In humans, no significant increase in the FIX level was observed with the same vector dose [10]. One possible explanation for the differences in these results is that the transduction efficiency of the type 2 AAV vectors into the skeletal muscles of humans differs from those in the animal models. Unpredicted adverse effects occurred in patients who received an AAV2 vector for FIX gene expression in the liver [9]. In this regard, a primate model may be required to more closely mimic the situation in humans, though non-human primate experiments may not reflect perfectly human situations.

Cynomolgus macaques are native to southern Asia and have been used as simian models in medical research, such as gene therapy studies for Parkinson's disease [26]. As reported previously, human FIX may be immunogenic in macaques under certain conditions, such as expression of human FIX in rhesus macaques with adenoviral vectors and cynomolgus macaques receiving repeated subcutaneous injections of human FIX in the presence of Freund's adjuvant [17,19]. Therefore, as long as antibodies against human FIX develop in macaques during transduction with vectors carrying the human FIX gene, long-term studies of human FIX expression will be impossible. In this context, FIX molecules that are less immunogenic in macaques may be suitable for long-term expression of FIX transgenes in macaques. Possible candidate FIX molecules for this purpose would be tagged macaque FIX or mutant macaque FIX proteins that can be distinguished from the native wild-type macaque FIX. Anti-FIX monoclonal antibodies were screened for their inability to bind to simian FIX. One antibody was identified, which forms the basis for an ELISA that can quantify human FIX in macaque plasma down to a concentration of 1.7 ng/mL (0.06% of the normal plasma FIX concentration) [18]. In the present study, we developed a mutant macaque FIX that can be detected accurately with this specific anti-human FIX monoclonal antibody.

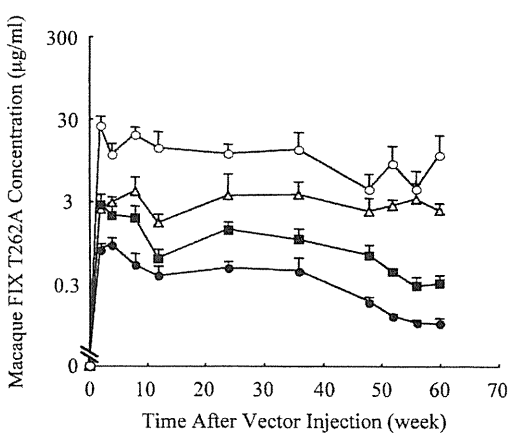

Fig. 3. Expression of macaque FIX T262A in mice using AAV8 vectors. The macaque FIX T262A levels in plasma of mice transduced with AAV8-HCRHAAT-macFIXT262A (open circles, 5×10^9 vector genome/g; open triangles, 5×10^8 vector genome/g) or AAV8-CMV-macFIXT262A (closed squares, 5×10^{10} vector genome/g; closed circles, 5×10^9 vector genome/g) were quantified by the 3A6-based ELISA.

Macaque plasma (%) (μg/ml)

Macaque FIX T262A (ng/ml)

Macaque FIX T262A Concentration (μg/ml)

Macaque FIX T262A is highly homologous to human FIX, with the amino acid sequence identity of 97.1% [16]. Among the 415 amino acid residues of mature FIX, 12 amino acid residues at 11 distinct positions of human FIX are different from those in macaque FIX [16]. Human FIX has two potential N-glycosylation sites, while macaque FIX has three potential N-glycosylation sites, similar to the case for murine FIX, porcine FIX, and bovine FIX. Of the three potential N-glycosylation sites in macaque FIX, two are located at the same positions as the sites in human FIX. Human FIX lacks the potential N-glycosylation site located at position 260 of macaque FIX [18]. Since Thr at position 262 was substituted with Ala in macaque FIX T262A, the consensus sequence Asn-X-Thr for N-glycosylation at this position was mutated, indicating that macaque FIX T262A may loose this potential N-glycosylation site. Asn 260 of macaque FIX may be glycosylated because macaque FIX T262A migrated faster than wild-type macaque FIX on Western blotting. Regarding the carbohydrate composition, macaque FIX T262A may be humanized, but has only a single amino acid substitution. Therefore, the amino acid sequence of macaque FIX T262A may be closer to wild-type macaque FIX than to human FIX. In terms of the coagulation activity of macaque FIX T262A, it has almost the same coagulation activity as wild-type macaque FIX and human FIX. This observation suggests that the macaque FIX T262A conformation may not be significantly altered and that the N-glycosylation at position N260 may not contribute significantly to the coagulation activity of macaque FIX.

We created macaque FIX with a FLAG sequence at the C-terminal end (FLAG-tagged macaque FIX) and analyzed its properties.

Recombinant FLAG-tagged macaque FIX expressed in HEK 293 cells was efficiently secreted from the cells and detected by both a polyclonal antibody against human FIX and anti-FLAG antibody M2. However, its coagulation activity was significantly decreased compared with wild-type macaque FIX, macaque FIX T262A and human FIX expressed in HEK 293 cells (data not shown). These data suggest that the conformation of FLAG-tagged macaque FIX is altered. It is also possible that the FLAG sequence is immunogenic in macaques. Therefore, macaque FIX T262A may be closer to wild-type macaque FIX than tagged macaque FIX.

The mice that had high macaque FIX T262A expression survived more than 1 year without any events such as a sudden death. However, effect of over expression of FIX on the thrombogenecity may need to be studied carefully and precisely with the AAV vectors carrying the mouse FIX gene, since there may be a significant species difference in interaction of coagulation factors. Macaques with high FIX expression by the vector used in this study might also be good models for studying the effect of supra-physiological FIX level on the coagulation system. These may be the future studies.

In conclusion, macaque FIX T262A bound to the specific anti-human FIX monoclonal antibody 3A6, was efficiently expressed after gene transfer to the liver *in vivo*, and was quantified by the 3A6-based ELISA in the presence of wild-type macaque FIX. Macaque FIX T262A may have advantages over human FIX for studying the long-term expression of transgene-derived FIX in macaques. Therefore, macaque FIX T262A may be useful as a tool for FIX gene transfer studies in macaques.

Conflict of interest statement

The authors declare that they had no affiliation with or financial involvement in any organization or entity with a direct financial interest in the subject matter or materials discussed in the manuscript.

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Impact of acute cellular rejection on coagulation and fibrinolysis biomarkers within the immediate post-operative period in pediatric liver transplantation

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Abstract: We studied restoration of the coagulation and fibrinolysis system in pediatric patients following liver transplantation and biomarkers of blood coagulation and fibrinolysis for suspecting the occurrence of acute cellular rejection. Coagulation activity recovered rapidly within two days following transplantation, but it took approximately 21–28 days for full recovery of the coagulation and fibrinolysis factors synthesized in the liver. PAI-1 levels were significantly higher in patients at the time of acute cellular rejection compared with levels after control of AR, and levels on days 14 and 28 in patients without AR. Plasma protein C and plasminogen levels at the time of rejection were significantly lower than those on day 14 in patients without AR. Statistical analysis suggested that an increase in plasma PAI-1 at a single time point in the post-operative period is a reliable marker among the coagulation and fibrinolysis factors for suspecting the occurrence of acute cellular rejection. These data suggested that appropriate anticoagulation may be required for 14 days after liver transplantation in order to avoid vascular complications and measurement of plasma PAI-1 levels may be useful for suspecting the occurrence of acute cellular rejection in pediatric patients following liver transplantation.

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Key words: liver transplant rejection – coagulation – fibrinolysis – plasminogen activator inhibitor 1

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Vascular thrombosis and immunological rejection of the transplanted liver in patients undergoing liver transplantation are frequent and serious post-operative complications (1). The majority of coagulation factors, factors regulating coagulation, and fibrinolysis factors are synthesized in the liver, and plasma coagulation factor levels may therefore fall for a short period after transplantation, but may return to normal levels upon regeneration of the grafted liver. Anastomosis of the vascular system of the grafted liver and the recipient vessels is carried out during liver transplantation, and anticoagulants are commonly administered for a period of time following surgery. However, restoration of the coagulation and fibrinolysis system following liver transplantation in pediatric patients has not been well studied (2, 3). In addition, the

Abbreviations: ADAMTS13, a disintegrin-like and metalloprotease with thrombospondin type 1 motif 13; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AR, acute rejection; AST, aspartate aminotransferase; ELISA, enzyme linked immunosorbent assay; LDH, lactate dehydrogenase; NAR, no acute rejection; PAI-1, plasminogen activator inhibitor 1; PELD, pediatric model for end-stage liver disease; PT-INR, prothrombin time-international normalized ratio; sES, soluble E-selectin; TM, thrombomodulin; vWF, von Willebrand factor; γ-GTP, γ-glutamyl transpeptidase.

thrombogenic state after liver transplantation is not well understood. We performed a single center study to investigate the coagulation and fibrinolysis system and the relationship between coagulation markers and acute cellular rejection following liver transplantation from living-related donors.

Materials and methods

Patients and study protocol

Sixty-three pediatric patients with liver failure due to biliary atresia (n = 59), ornithine transcarbamidase deficiency (n = 2), or Wilson's disease (n = 2) underwent living-related liver transplantation from April 2001 to March 2006 and were enrolled in this study. Most of the patients with biliary atresia had previously undergone hepatic porta-jejunostomies.

Description of patients

The patients were classified into two patient groups: one with acute cellular rejection (group AR, n = 24) and one with no acute cellular rejection (group NAR, n = 39). The diagnosis of acute cellular rejection was made by liver biopsy. There were no significant differences between group AR and group NAR in terms of age, gender, basal diseases, or the use of calcineurin inhibitors (data not shown). The PELD scores (AR, 13.0 ± 7.8; NAR, 15.1 ± 9.5), the amount of blood loss (AR, 85.0 ± 127.8 mL/kg; NAR, 125.8 ± 176.0 mL/kg), the amount of total blood transfusion (AR, 162.1 ± 109.1 mL/kg; NAR, 161.8 ± 170.8 mL/kg), the amount of plasma transfusion (AR, 64.1 ± 49.1 mL/kg; NAR, 94.2 ± 89.1 mL/kg), the cold ischemic time of graft liver (AR, 149.75 ± 126.4 min; NAR, 121.1 ± 69.1 min), and the warm ischemic time of graft liver (AR, 64.9 ± 18.1 min; NAR, 65.1 ± 13.3 min) upon operation were not significantly different between group AR and group NAR. Patients with severe infections or major bleeding episodes at the time of blood sampling for analysis were excluded from the analysis.

Immunosuppression and anticoagulation protocols

The standard protocol for immunosuppression was as follows. Both methylprednisolone and a calcineurin inhibitor (tacrolimus or cyclosporine) were used for immunosuppression. Intravenous administration of methylprednisolone (20 mg/kg) was started during the operation and the dosage was tapered to 3 mg/kg on day 1 and to 0.5 mg/kg on day 7 after liver transplantation. A calcineurin inhibitor was infused intravenously after transplantation and the blood concentration of tacrolimus or cyclosporine was adjusted to 18–20 ng/mL or 200–300 ng/mL till day 7 after liver transplantation, respectively. Intravenous injection of calcineurin inhibitor and methylprednisolone were converted to oral administration of these regimens after patient's oral intake had been fully confirmed and the blood concentration of tacrolimus or cyclosporine was adjusted 10–15 ng/mL or 100–150 ng/mL, respectively. The methylprednisolone dose was tapered to 0.06 mg/kg on day 30. Post-operative anticoagulation was performed with intravenous administration of dalteparin (low molecular weight heparin) at the dose of 2 U/kg/h, nafamostat mesilate (serine protease inhibitor with anticoagulant activity) at the dose of 0.1 mg/kg/h, and prostaglandin E1 at the dose of 0.01 μg/kg/min till day 7 after transplantation. Anticoagulation was continued with intravenous administration of heparin (unfractionated heparin) at the dose of 8 U/kg/h from day 8 to day 21 after liver transplantation.

Blood sample collection and analysis

All samples were obtained from patients with informed consent, according to the Declaration of Helsinki. Routine laboratory tests including complete blood counts, coagulation tests, blood chemistry analysis, and urinalysis were performed, and biomarkers of blood coagulation and fibrinolysis, i.e., PAI-1, TM, ADAMTS13, and sES were measured before and after liver transplantation on days 1, 3, 7, 10, 14, 21, and 28. Blood sampling was performed on days 35 and 49 in some patients. These were quantified using commercially available ELISAs (Mitsubishi Chemical Medience Co., Tokyo, Japan; Diaclone, Tepnel Research Products & Services, Cedex, France) (4, 5). The plasma activity levels of plasminogen and protein C were quantified using laboratory test kits (Siemens Healthcare Diagnostics Inc., Deerfield, IL, USA). Rationale for measurements of these biomarkers are as follows. PT-INR is currently used worldwide as a coagulation test to monitor the effects of anticoagulants such as coumarin in patients at risk of thrombosis. Protein C is a vitamin K-dependent protein synthesized in the liver that functions as an important regulatory factor for coagulation (6). Plasminogen is the zymogen of plasmin, a key enzyme in fibrinolysis, and is also synthesized in the liver (7). Therefore, plasma protein C and plasminogen levels were thought to be good markers for the restoration of the coagulation and fibrinolysis system following liver transplantation. Levels of these markers might correlate with protein synthesis in the liver, thereby reflecting regeneration of the graft liver. Additionally, measurement of these factors may also be important for patient management, because deficiency of protein C and type II plasminogen deficiency are thought to increase the risk of thrombosis (7, 8). The fibrin degradation product level, determined by the monoclonal antibody specific for degradation products of cross-linked fibrin, is a biomarker for the presence of a thrombus and is used to diagnose venous thrombosis and disseminated intravascular coagulation, however, the fibrin degradation product level may be affected by the presence of blood clots in the extravascular spaces (e.g., the peritoneal cavity), and may therefore not accurately reflect the thrombogenic state in the post-operative period. Thus, the soluble fibrin level was used to assess the thrombogenic state during the post-operative period following liver transplantation. PAI-1 is a primary regulator of fibrinolysis that is synthesized mainly in endothelial cells. Plasma PAI-1 levels change significantly in various pathological conditions (4). ADAMTS13 is the vWF cleaving protease that plays an important role in vWF multimer processing (9). It is synthesized in liver stellate cells and the liver is thought to be the primary source of ADAMTS13 in the circulation (9–11). In addition to the liver stellate cells, vascular endothelial cells in other organs may also be able to synthesize ADAMTS13 (12), and ADAMTS13 mRNA has been detected in the liver, kidneys and lungs in mice (13). ADAMTS13 deficiency results in platelet thrombus formation in the circulation, resulting in the development of a typical thrombotic microangiopathy (9). It is possible that ADAMTS13 deficiency might occur after liver transplantation, and plasma ADAMTS13 levels in patients were

therefore quantified following transplantation. TM, an important regulator of blood coagulation, is synthesized in vascular endothelial cells and is used as a marker of vascular injury (6). The sES level has been used as a marker for endothelial cell dysfunction (14). For example, the sES level is increased in systemic infections such as sepsis.

Diagnosis of acute cellular rejection

The diagnosis of acute cellular rejection was made by liver biopsy and was evaluated using the rejection activity index (3) scores (1, 15, 16). Patients suspected of suffering from acute cellular rejection because of deterioration of liver function (increased serum levels of bilirubin, AST, ALT, ALP, LDH, and γ-GTP compared with previous levels) were subjected to ultrasonography-guided liver biopsy. The liver biopsy specimens were examined for the presence of acute cellular rejection. Patients diagnosed with acute cellular rejection were subjected to intensive immunosuppressive therapy with intravenous methylprednisolone. Mycophenolate mofetil and/or OKT3 were also administered in some patients. Plasma samples obtained before starting administration of the intensive immunosuppressive regimens were evaluated in the following studies.

Statistical analysis

Statistical analyses were performed using SPSS software (SPSS Inc., Tokyo, Japan). Student *t*-tests were used to compare the mean values between groups. Multiple logistic regression analysis was used to investigate the association between biomarkers of blood coagulation and fibrinolysis and the occurrence of acute cellular rejection. p-values < 0.05 were considered statistically significant.

Results

Analysis of the coagulation and fibrinolysis system following liver transplantation

Changes in mean values of coagulation tests in patients without acute cellular rejection, vascular complications, or severe infections are shown in Fig. 1. The coagulation activity after liver transplantation was assessed by measuring prothrombin time (PT-INR). The mean PT-INR value rose to approximately 1.8 on day 1, but quickly fell again to < 1.5 on day 2, and then normalized gradually. These data suggest that the coagulation activity rapidly recovered after transplantation, once the graft liver started to function.

The mean protein C level of patients before liver transplantation decreased to 57.5% of the normal level. This may have been due to the decreased synthesis of protein C in the liver because most patients had liver failure. The mean protein C level fell to approximately 50% of the normal level on day 1 post-transplantation, and then increased gradually, reaching ≥80% of the normal level by day 14. The mean plasminogen level changed in a similar manner to protein C. By day 28, both protein C and plasminogen levels had returned to almost 90–100% of the normal levels. The nadir values of protein C and plasminogen on day 1 post-transplantation might

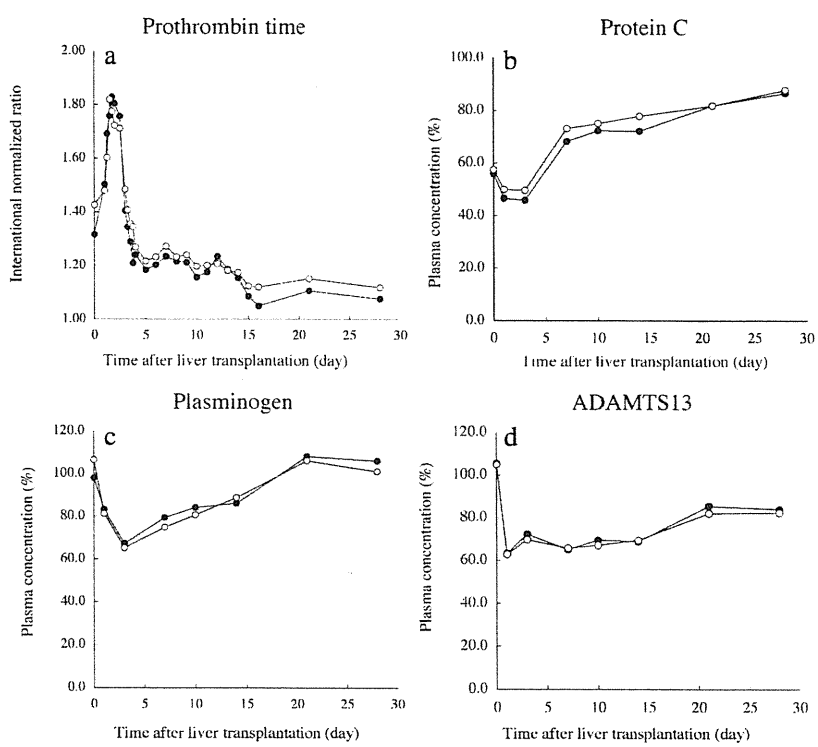

Fig. 1. Restoration of the coagulation and fibrinolysis system following liver transplantation. (a) The mean values of coagulation activity assessed by PT-INR and (b) plasma levels of protein C (normal range 67.1–129.0%), (c) plasminogen (normal range 85.0–120.0%), and (d) ADAMTS13 (normal range 100 ± 15%) of patients without complications (open circle) and of patients with acute cellular rejection (closed circle) are shown.

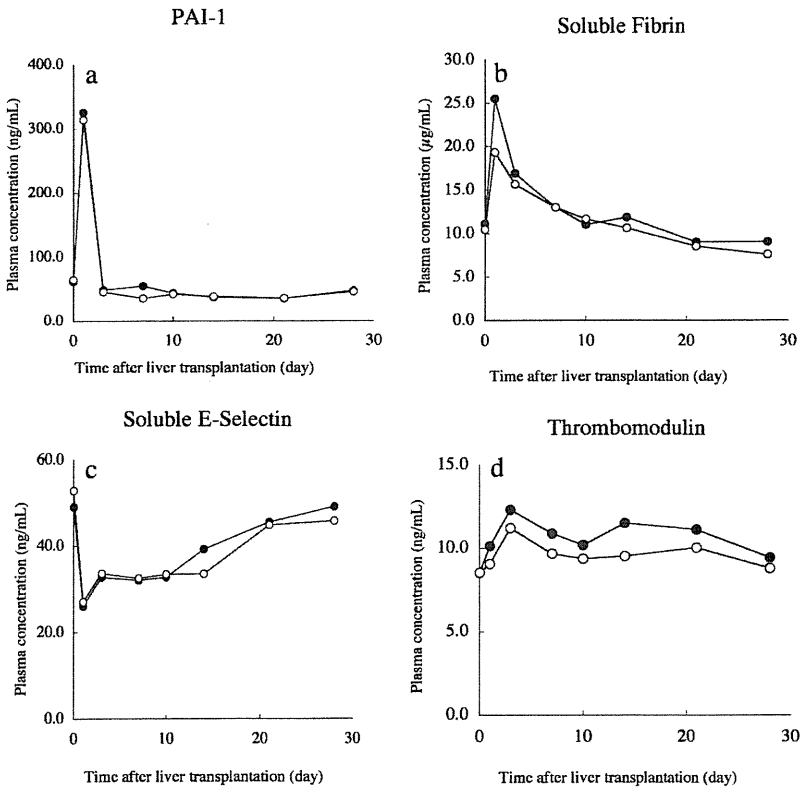

Fig. 2. Analysis of biomarkers of coagulation and fibrinolysis following liver transplantation. The mean plasma levels of PAI-1 (normal range 20–30 ng/mL), soluble fibrin (normal range: < 7.5 μg/mL), sES (normal range: < 37.5 ng/mL), and TM (normal range 4.46 ± 1.36 ng/mL) of patients without complications (open circle) and of patients with acute cellular rejection (closed circle) are shown.

be affected by plasma transfusion during and after surgery. These data suggest that the synthesis of coagulation factors in the graft liver may start on day 1, resulting in rapid recovery of coagulation activity, but it may take up to 14 days for recovery of the coagulation and fibrinolysis system to near normal levels, and 21–28 days for full restoration of the system after liver transplantation. These data also suggest that graft livers may regenerate to the appropriate size within four wk, though graft livers may vary in size depending on their recipients and donors. The average levels of the coagulation and fibrinolysis factors in patients with acute cellular rejection was not significantly different from those in patients without acute cellular rejection in the post-operative periods, but rate of restoration of the protein C and plasminogen levels on day 14 in group AR was slow.

Soluble fibrin levels in patients with no complications increased significantly on day 1 and then gradually decreased, normalizing by day 14 as shown in Fig. 2. These data suggest that the thrombotic state may continue for 14 days after liver transplantation, and that appropriate antithrombotic therapy may therefore be required during this period.

The mean plasma PAI-1 level was increased approximately 10-fold on post-operative day 1, compared with the normal level, but returned quickly to the normal level on day 3 after transplantation (Fig. 2). These data, together with the changes in the plasminogen level during the post-operative period (Fig. 1), suggest that fibrinolysis activity was suppressed on day 1 after liver transplantation.

The average plasma ADAMTS13 level decreased significantly on day 1 post-transplantation (Fig. 2), but the decrease was not as severe as that of protein C or plasminogen (Fig. 1). However, low levels of ADAMTS13 were maintained for 14 days after liver transplantation. These changes in plasma ADAMTS13 levels after liver transplantation did not parallel those of protein C or plasminogen (Fig. 1), reflecting the extrahepatic synthesis of ADAMTS13 and the possibility that ADAMS13 is synthesized not in hepatocytes, but in stellate cells in the liver. The plasma ADAMTS13 level fell to 28.4% of the normal level in one patient, but she showed no typical signs of thrombotic microangiopathy.

The TM level was increased on day 3 post-transplantation and remained at the upper limit of the normal range after day 7 (Fig. 2). The sES level was significantly increased in patients before liver transplantation (Fig. 2), which may be explained by the fact that many patients enrolled

in the study had undergone hepatic porta-jejunostomies and therefore had biliary tract infections before transplantation. The sES level was reduced post-transplantation, and remained almost within the normal range until day 14, but then was significantly increased on days 21 and 28 (Fig. 2). This increase in the sES level was not associated with the presence of infection or other disease states. The average changes of biomarkers of the coagulation and fibrinolysis system in patients with acute cellular rejection was not significantly different from those in patients without acute cellular rejection in the post-operative periods.

Three patients in this study suffered from hepatic artery thrombosis after liver transplantation, and an increased PT-INR (prolongation of prothrombin time) was detected in all three patients. Increase of plasma PAI-1 in the following samples of two patients was observed. Decrease of plasma protein C and plasma ADAMTS 13 in the following samples were observed in two patients. Other biomarkers did not change significantly. However, due to the small sample size, the predictive value of this test for the development of vascular complication was inconclusive.

Relationship between coagulation and fibrinolysis markers and acute cellular rejection

Patients were divided into two groups, group AR and group NAR, based upon the presence of acute cellular rejection as described above. The mean onset time of acute cellular rejection in group AR was on day 15 ± 8.7 after liver transplantation, while the mean time for data collection was on day 14 ± 7.9. Laboratory data and coagulation markers for each group at two time points were subjected to statistical analysis. Measurements taken immediately before the diagnosis of acute cellular rejection in group AR were compared with those taken after the cessation of rejection by intensive treatment with methylprednisolone in group AR, and those taken on days 14 and 28 in group NAR.

Statistical analysis of the mean levels of coagulation and fibrinolysis markers (Table 1) revealed that the PAI-1 level at the time of acute cellular rejection in group AR was significantly higher than that after cessation of rejection in group AR, and those on days 14 and 28 in group NAR (data for day 28 of group NR are not shown in Table 1).

The plasma protein C and plasminogen levels at the time of AR diagnosis in group AR were significantly lower than those on day 14 in group NAR.

Table 1. Coagulation and fibrinolysis biomarkers following liver transplantation

	Group AR (n = 24)		Group NAR (n = 39)
	Before*	After†	Day 14
PAI-1 (ng/mL)	79.3 ± 103.9‡	23.0 ± 10.7	38.5 ± 30.4
Plasminogen (%)	85.2 ± 22.8‡	99.4 ± 29.0	97.68 ± 13.8
Protein C (%)	65.7 ± 23.0‡	89.3 ± 37.9	87.2 ± 25.5
ADAMTS13 (%)	67.5 ± 24.1	77.8 ± 23.6	72.5 ± 17.4
ATIII (%)	96.3 ± 17.3	111.5 ± 57.4	99.3 ± 14.9
PT-INR	1.17 ± 0.21	1.08 ± 0.13	1.13 ± 0.13
Fibrinogen (mg/mL)	295.3 ± 116.4	296.3 ± 106.7	280.6 ± 74.0
Thrombomodulin (U/mL)	10.2 ± 3.8	10.8 ± 4.8	8.7 ± 5.2
Soluble E-selectin (μg/mL)	43.8 ± 16.7	46.4 ± 19.0	33.5 ± 17.2
Soluble fibrin (μg/mL)	13.57 ± 17.3	8.64 ± 14.9	10.2 ± 13.9

*Values at the time immediately before acute cellular rejection.
†Values after cessation of acute cellular rejection.
‡Values taken from the time point proximate to acute cellular rejection (before) are significantly different from those of group AR after cessation of acute cellular rejection (after) and those on day 14 in group NAR ($p < 0.01$).
Values are mean ± s.d.

The ADAMTS13 level at the diagnosis of AR in group AR appeared to be lower than that after cessation of rejection in group AR, and those on day 14 in group NR, though the differences were not statistically significant.

There were no significant differences between the levels of other coagulation and fibrinolysis markers in patients at the time of rejection diagnosis and after cessation of acute cellular rejection in group AR, or the levels on days 14 and 28 in group NAR.

The changes of the coagulation and fibrinolysis factors and biomarkers before the diagnosis of acute cellular rejection by liver biopsy were studied. These biomarkers levels of samples obtained from the patients proximate to the diagnosis of acute cellular rejection (AR-proximate sample in Fig. 3) were compared with those obtained before the AR-proximate sample (earlier sample in Fig. 3). The PAI-1 level in the AR-proximate samples were significantly higher than that in the earlier samples. The mean values of protein C, plasminogen, and ADAMTS13 in the AR-proximate samples was expected to be higher than those in the earlier samples, but they were lower than the earlier samples though the differences were not statistically significant. The mean values of other biomarkers in the two time points were not significantly different.

Multiple logistic regression analysis was performed to identify the coagulation and fibrinolysis markers for suspecting the occurrence of acute cellular rejection. Absolute values of coagulation and fibrinolysis factors (protein C, plasminogen, ADAMTS13) synthesized in the liver

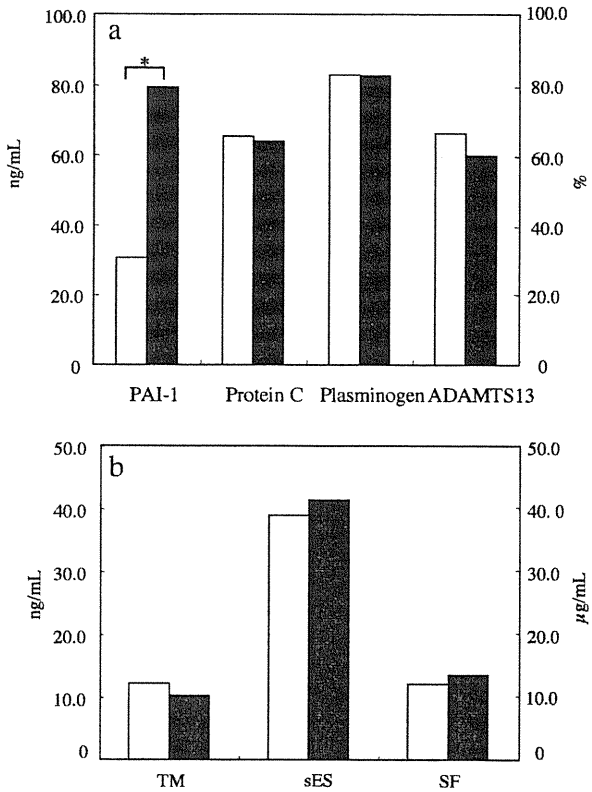

Fig. 3. Changes of coagulation and fibrinolysis factors and biomarkers in patients with acute cellular rejection. The mean plasma levels of coagulation and fibrinolysis factors and biomarkers obtained from patients with acute cellular rejection at two time points were shown. The AR-proximate samples (closed square) were obtained from the patients proximate to the diagnosis of acute cellular rejection. The earlier samples (open square) were obtained before the AR-proximate samples.

were difficult to ascertain using this method, and the changes in levels between time points were therefore analyzed. PAI-1 levels are independent of regeneration of the liver and an increase in plasma PAI-1 levels of >40 ng/mL at a single time point was therefore taken into account. Increases in soluble fibrin, TM, or sES since the previous time point, and above the normal range, were also taken into account. A summary of the multiple logistic regression analysis of coagulation and fibrinolysis markers is shown in Table 2. These data suggest that an increase in PAI-1 levels, and a decrease in protein C, plasminogen, or ADAMTS13 levels, were independently related to the occurrence of acute cellular rejection. Other markers were not related to the occurrence of acute cellular rejection (Table 2). Among these markers, an increase in plasma PAI-1 levels was observed in almost 80% of the patients in group AR.

Table 2. Multiple logistic regression analysis of biomarkers

	Odds ratio	Confidence interval	p-value
PAI-1	17.91	4.89–64.36	<0.001
ADAMTS13*	6.40	1.85–22.03	0.003
Protein C*	4.58	1.14–18.28	0.027
Plasminogen*	7.86	1.23–49.83	0.02
Soluble fibrin†	0.60	Not applicable	0.60
Soluble E-selectin†	0.60	Not applicable	0.65
Thrombomodulin†	0.421	Not applicable	0.42

*Decrease of marker values from the previous time point was adopted.
†Increase of marker values from the previous time point was adopted.

Discussion

The prevention and treatment of vascular thrombosis and immunological rejection of the transplanted liver during the post-operative period is a keystone of patient management. The present study analyzed the coagulation and fibrinolysis system following liver transplantation in pediatric patients to identify biomarkers for suspecting the occurrence of acute cellular rejection.

The present study suggests that the coagulation activity recovered rapidly once the graft liver started functioning, and that the graft liver might regenerate to the appropriate size in 21–28 days, with coincident full recovery of the coagulation and fibrinolysis system in pediatric patients undergoing liver transplantation. The present study also suggests that the hypercoagulable state persisted for 14 days after surgery, and that appropriate anticoagulation may therefore be required at least for 14 days post-transplant, even in the absence of any apparent vascular complications.

Recent advances in the management of patients with liver transplants have improved the clinical outcome of these patients. Adjustments in the doses of immunosuppressive drugs such as calcineurin inhibitors, based on their blood concentrations, are widely conducted after liver transplantation. However, immunological rejection of the transplanted liver still develops in a certain ratio of these patients, even when the blood calcineurin inhibitor concentration is within the appropriate therapeutic range (17, 18). A variety of methods for evaluating immune cell activation have been proposed as a basis for adjusting immunosuppressive therapy, and these have been shown to be useful for assessing the level of immunosuppression (19–22). Intensive treatment of acute cellular rejection with high dose methylprednisolone, with or without other medicines such as OKT3, is usually effective, though the prediction and rapid diagnosis of AR may be important for its effective treatment. In

this regard, the timely suspicion of acute cellular rejection using laboratory markers is a key indicator of the need for liver biopsy. Fluorescent-activated cell sorting analysis of CD25, CD28, and CD38 expression in peripheral lymphocytes is considered to be useful, not only for evaluation of the degree of immunosuppression, but also for the prediction of acute allograft cellular rejection (22). The present study showed that four coagulation and fibrinolysis markers, i.e., increase in PAI-1, decrease in protein C, decrease in plasminogen, and decrease in ADAMTS13, might be used as markers for suspecting the occurrence of acute cellular rejection. Statistical analysis suggested that an increase in the plasma PAI-1 level was the most reliable and sensitive marker for acute cellular rejection. Protein C, plasminogen, and ADAMTS13 are all synthesized in the liver, and their levels may therefore depend on the size and regeneration of the graft liver, and their plasma levels at any given time point might thus be less reliable as predictors of acute cellular rejection. PAI-1 is synthesized mainly in the vascular endothelial cells and its plasma level was elevated on day 1 after liver transplantation, and had returned to pretransplant levels after day 3. An increased plasma PAI-1 level at a single time point after day 1, together with a deterioration in liver function, may therefore be adopted as a predictive marker for acute cellular rejection.

Acute cellular rejection is characterized by portal inflammation, bile duct inflammation, and subendothelial cell inflammation (15, 16). Recent studies have suggested that not only T-cells, but also B-cells, are involved in acute cellular rejection, and cytokines and chemokines may also play roles in this process (23). As shown in a previous report, Toll-like receptor signaling through MyD88 may be involved in acute allograft rejection, indicating that toll-like receptors may be activated in the transplant setting causing inflammatory cytokine release (24). Therefore, the increase in PAI-1 levels seen during acute cellular rejection may be accounted for by immune cell-derived cytokine/chemokine activation of, and inflammation of, sinusoid-endothelial and portal vein endothelial cells. An increased PAI-1 level has previously been shown to be predictive for veno-occlusive disease developing after bone marrow transplantation (25), and this mechanism is thought to be responsible for busulfan-related toxic injury of sinusoidal endothelial cells (26, 27). The increase in plasma PAI-1 levels in patients with allograft cellular rejection is not as high as that seen in veno-occlusive disease, suggesting that the mechanisms and the outcomes of these PAI-1 increases may differ. Although the mechanisms of activation of endothelial cells may differ in veno-occlusive disease and in acute cellular rejection after allograft liver transplantation, both might result in increased plasma levels of PAI-1. Further studies are required to determine the precise mechanism responsible for the increase in PAI-1 levels occurring during acute cellular rejection.

Cytokines released from infiltrated immune cells in the liver, and inflammation in portal and sinusoid endothelial cells, might also inhibit the synthesis of ADAMTS13 in stellate cells, resulting in decreased plasma ADAMTS13 levels because the plasma ADAMTS13 level was significantly decreased in patients with sepsis-induced disseminated intravascular coagulation (5) and ADAMTS13 mRNA expression in the liver is decreased in endotoxin-injected mice (13). The decrease in protein C and plasminogen levels associated with acute cellular rejection might be due to their reduced synthesis by the graft hepatocytes, and a reduction in levels of these markers might therefore take time to become apparent. The decrease in plasminogen levels in patients with acute cellular rejection was less severe than that in protein C levels. These differences may be due to differences in the plasma half-lives of these molecules.

In conclusion, we have performed a comprehensive analysis of the coagulation and fibrinolysis system in pediatric patients undergoing orthotopic liver transplantation. Coagulation activity was quickly normalized by two days after liver transplantation. However, it took for 21–28 days for full restoration of the coagulation and fibrinolysis system. The post-operative thrombogenic state continued for approximately 14 days. PAI-1 may be used as predictive markers for acute cellular rejection in pediatric patients. These findings might also be applicable to adult liver transplant patients, though this needs to be confirmed by future prospective studies.

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A Phase I Study of Aromatic L-Amino Acid Decarboxylase Gene Therapy for Parkinson's Disease

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Gene transfer of dopamine-synthesizing enzymes into the striatal neurons has led to behavioral recovery in animal models of Parkinson's disease (PD). We evaluated the safety, tolerability, and potential efficacy of adeno-associated virus (AAV) vector–mediated gene delivery of aromatic L-amino acid decarboxylase (AADC) into the putamen of PD patients. Six PD patients were evaluated at baseline and at 6 months, using multiple measures, including the Unified Parkinson's Disease Rating Scale (UPDRS), motor state diaries, and positron emission tomography (PET) with 6-[^{18}F]fluoro-L-*m*-tyrosine (FMT), a tracer for AADC. The short-duration response to levodopa was measured in three patients. The procedure was well tolerated. Six months after surgery, motor functions in the OFF-medication state improved an average of 46% based on the UPDRS scores, without apparent changes in the short-duration response to levodopa. PET revealed a 56% increase in FMT activity, which persisted up to 96 weeks. Our findings provide class IV evidence regarding the safety and efficacy of AADC gene therapy and warrant further evaluation in a randomized, controlled, phase 2 setting.

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INTRODUCTION

Dopamine replacement has been the standard pharmacotherapy for motor impairment in Parkinson's disease (PD). Although virtually all patients benefit from levodopa at an early stage of the disease, severe loss of nigrostriatal nerve terminals in advanced PD leads to profoundly decreased activities of dopamine-synthesizing enzymes, including aromatic L-amino acid decarboxylase (AADC), an essential enzyme that converts levodopa to dopamine. Failure to respond to levodopa therapy may result from a reduction in AADC activity, decreased dopamine storage capacity in synaptic vesicles, postsynaptic changes in striatal output neurons, and abnormalities of nondopaminergic neurotransmitter systems.[1,2] Systemic administration of high-dose levodopa enhances oscillations in motor performance and complications, including hallucinations, due to dopaminergic stimulation of the mesolimbic system.

One potential treatment for advanced PD is gene therapy to restore striatum-selective dopamine production. In addition to AADC, tyrosine hydroxylase, which converts L-tyrosine to levodopa, and guanosine triphosphate cyclohydrolase I, which catalyzes biosynthesis of the essential tyrosine hydroxylase cofactor, tetrahydrobiopterine, are necessary for efficient synthesis of dopamine.[2] Viral vector–mediated gene transfer of these dopamine-synthesizing enzymes has been shown to achieve behavioral recovery in animal PD models, with efficient transduction of striatal neurons that escape degeneration.[3–6] When tyrosine hydroxylase and guanosine triphosphate cyclohydrolase I are expressed in the striatum, levodopa can be synthesized continuously. This strategy would be useful for reducing motor fluctuations associated with intermittent levodopa intake. Gene transfer of AADC alone in combination with oral levodopa administration would be a safer strategy for initial clinical trials. In the latter approach, the patients still need to take levodopa to control motor symptoms, but excess production of dopamine could be avoided by reducing the dose of levodopa. We assessed the safety, tolerability, and the potential efficacy of intraputaminal infusion of recombinant adeno-associated virus (AAV) serotype 2 vector encoding human AADC (AAV-hAADC-2) in patients with mid- to late-stage PD. We also examined whether the short-duration response to levodopa, the antiparkinsonian response that parallels the plasma levodopa levels, would change after gene therapy.[7]

RESULTS

Patient disposition and baseline characteristics

Six patients (4 men, 2 women), mean age 60 (range, 51–68) years, were enrolled (Table 1). The mean disease duration was 10 (range, 5–18) years, and time on levodopa was 9.3 (range, 5–15) years. The average baseline daily levodopa and levodopa equivalent doses were 642 and 808 mg, respectively.

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Table 1 Patients' baseline characteristics

Subject	Age (years)	Sex	Disease duration (years)	Time on levodopa (years)	Levodopa dose (mg)	Levodopa equivalents (mg)
A-1	51	M	11	9	600	900
A-2	63	M	9	9	450	650
A-3	66	F	7	7	500	700
A-4	58	M	11	11	700	700
A-5	68	F	18	15	1,000	1,100
A-6	56	M	5	5	600	800
Mean (SD)	60 (6.5)	67% M	10 (4.5)	9.3 (3.4)	642 (196)	808 (169)

Abbreviations: F, female; M, male.
Patients are listed in the order in which they received treatment. Levodopa equivalents were estimated as follows: 100 mg of levodopa with a dopa-decarboxylase inhibitor is equivalent to 0.8 mg talipexole, 1 mg pergolide, 1 mg pramipexole, and 1.5 mg cabergoline.

Primary end point

The procedure was well tolerated. All patients completed all protocol-defined visits. One patient (patient A-2) had a venous hemorrhage in the right frontal lobe just below a burr hole that was found on CT scan 3 days after infusion. The patient used his left arm less frequently than his right arm for 3 weeks; this was assumed to reflect mild frontal lobe dysfunction and resolved completely. Mild, transient headache around the burr holes was present for 2 days after surgery in all patients. There were no significant laboratory test abnormalities. All patients had mildly increased titers of anti-AAV2-neutralizing antibodies 6 months after treatment, which tended toward baseline concentrations thereafter (Table 2).

Clinical evaluations

The clinical results are summarized in Table 3. Intraputaminal AAV-hAADC-2 infusion significantly improved both total and motor scores of the unified Parkinson's disease rating scale (UPDRS) in the OFF state. Five of six patients showed substantial improvement in UPDRS motor ratings in the OFF state (Figure 1). Changes in the UPDRS ON state and the percent of ON state hours in a day were not significant. One patient with relatively mild motor symptoms at baseline did not improve on UPDRS (A-3 in Figure 1). However, this patient showed a remarkable increase in mobile time as measured by the diaries (28% at baseline to 58% at 6 months after gene transfer; Figure 2). The daily dose of levodopa was unchanged in two patients (A-2 and A-5) and reduced in three patients (A-1, A-3, and A-5) at 6 months. Patient A-6, who had daytime sleepiness, preferred to reduce pramipexole instead of levodopa after gene therapy.

The last three patients underwent the levodopa test after our institutional review board confirmed the safety of AADC gene transfer in the first three patients. The short-duration response to levodopa did not change significantly after gene therapy in these three patients, though UPDRS motor scores at 6 months showed slight improvement at 30 minutes in patient 5 and at 120 minutes in patient 4 after levodopa intake (Figure 3). Significantly higher peak plasma levodopa concentrations were observed in these two patients after gene therapy.

The mini-mental state examination (MMSE) and geriatric depression scale (GDS) scores did not change significantly.

Table 2 Changes in neutralizing AAV2 antibody titers in sera following gene therapy

Subject	Pre	2 weeks	6 months	1 year
A-1	1:2	1:4	1:4	1:4
A-2	<1	1:32	1:4	1:2
A-3	1:32	1:64	1:64	1:32
A-4	1:32	1:32	1:256	1:64
A-5	1:4	1:32	1:32	1:32
A-6	<1	1:16	1:32	1:32

Abbreviations: AAV, adeno-associated virus.
Titers are determined by *in vitro* assay and represented as "1:" dilutions.

Table 3 Clinical outcomes of six patients

	Baseline	6 months	*P* value
UPDRS Total OFF	53 (12.4)	38 (10.1)	0.049*
UPDRS Total ON	15 (7.2)	10.7 (2.9)	0.262
UPDRS Part III (Motor) OFF	25.3 (9.4)	13.7 (6.0)	0.024*
UPDRS Part III (Motor) ON	5.2 (4.6)	1.8 (1.5)	0.120
Percent day spent in mobile state	48.8 (12.9)	55.4 (14.8)	0.348
Daily levodopa equivalents dose, mg	808 (169)	707 (233)	0.097

Abbreviations: OFF, off-medication state; ON, on-medication state; UPDRS, Unified Parkinson's Disease Rating Scale.
Data are presented as means (SD). The UPDRS scores in each patient did not change during the 2 months of the screening period.
*$P < 0.05$.

PET analysis

PET imaging revealed increased 6-[^{18}F]fluoro-L-*m*-tyrosine (FMT), a tracer for AADC, activity 4 weeks postoperatively, which persisted at 6-month evaluation (Figure 4). The mean increase in FMT uptake from baseline in the combined (right and left) putamen at 24 weeks was 56%. Two patients (A-1 and A-2) who had PET scans 96 weeks after surgery showed persistently increased FMT uptake. In these two patients, motor performance in the OFF state also maintained its improvement at 96 weeks.

DISCUSSION

Extensive preclinical studies on both rodent and nonhuman primate models of PD have shown that AAV vectors can express exogenous genes for a long time in the brain target areas without

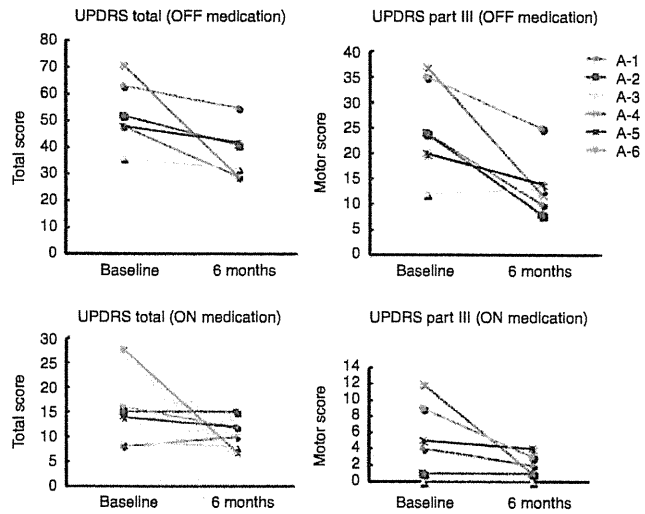

Figure 1 Changes in UPDRS scores. Absolute changes in scores from baseline to 6 months for individual patients. OFF, off-medication state; ON, on-medication state; UPDRS, Unified Parkinson's Disease Rating Scale.

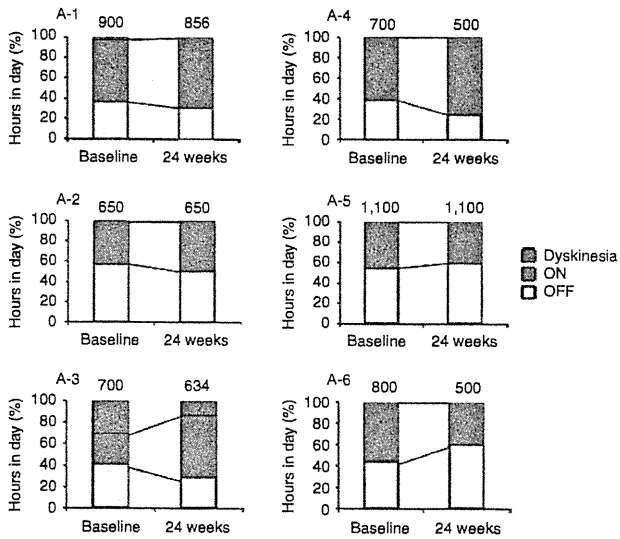

Figure 2 Evaluation of patients' diaries and daily doses of levodopa equivalents. For each 30-minute interval throughout the day, the patients recorded whether they were mobile (ON), immobile (OFF), or asleep. They also recorded the time with troublesome dyskinesias (Dyskinesia). The graph shows the percentage of hours in a day spent in each condition at baseline and at 6 months. The numbers on the bars indicate the mean daily doses of levodopa equivalents (mg). OFF, off-medication state; ON, on-medication state.

significant toxicity.[3,4,6,8,9] Recently, three phase I clinical trials of gene therapy for advanced PD demonstrated that AAV vector-mediated gene delivery into the subthalamic nucleus or putamen was safe and tolerable.[10–13] In this study, the safety of the AAV vectors for clinical use in the human brain was confirmed. Although one patient developed a venous hemorrhage in the subcortical white matter along the trajectory, it is well known that cerebral bleeding occasionally occurs in association with surgical procedures for deep brain stimulation in which electrodes are inserted into the basal ganglia through the frontal lobe white matter.[14,15] PET imaging in this patient showed that putaminal AADC expression was not affected by the subcortical venous hemorrhage and persisted up to 96 weeks. Thus, the venous hemorrhage was probably due to the surgical procedure and not gene transduction.

Although the present trial was a small, open-label study, and the nonblinded, uncontrolled analysis limits the interpretation, the initial efficacy outcomes are encouraging. Our patients showed improved motor performance in the OFF state. Levodopa has a relatively short plasma half-life (60–90 minutes), and antiparkinsonian effects observed after levodopa administration have generally been recognized as short- and long-duration responses. The short-duration response roughly parallels the plasma levodopa concentrations and is thought to be closely linked to dyskinesia, whereas the long-duration response builds up over weeks and improves trough (worst) motor performance in the OFF state.[7] Because the pattern of the short-duration response to levodopa did not change after gene therapy in our patients, the beneficial effect on the OFF state appears to be attributed to augmentation of the long-term response to levodopa.[16] In the preclinical studies with animal models of PD, AAV vectors mainly transduced medium spiny neurons that have dopamine receptors, and extracellular dopamine was increased in the striatum after administration of levodopa.[5,17] The mechanism underlying the long-duration response is not sufficiently understood, and future study is necessary to determine how nonphysiologic production of dopamine in the striatal neurons could enhance the response. It has been reported that the sustained long-duration response to levodopa is greater in patients treated with higher single doses of levodopa.[18] Thus, it is likely that increased dopamine in the putamen after gene transfer may enhance the stable long-duration response. Motor fluctuations in PD are associated with increased response to levodopa with a deeper trough in motor performance, rather than shortening of the response. Improving trough or OFF state motor function by augmenting the long-term response would likely reduce motor fluctuation.[16] Two of three patients in whom the short-duration response to levodopa was studied showed increased peak plasma levodopa concentrations after gene therapy. This finding may simply reflect variable absorbance of levodopa, and it remains to be elucidated whether changes in gastrointestinal absorption could be related to better motor performance in the OFF state.[19]

Activities and levels of AADC mRNA and protein are profoundly reduced in advanced PD,[2] but there are still several types of AADC-containing cells in the striatum, such as serotonin neurons, intrinsic dopamine neurons, AADC-containing "D" neurons, and glial cells.[20] These cells may act as a local source of dopamine. However, dopamine produced in nondopamine cells may not be taken up into dopamine cells and stored in synaptic vesicles, as dopamine transporter and vesicular monoamine transporter 2 are also reduced in advanced PD. The functional efficacy of dopamine produced from exogenous levodopa in these cells may be limited, at least in primates.[2,3] Striatal output neurons, main targets in AADC gene therapy, play a principal role in dopamine modulation of motor function in the basal ganglia. Dopamine synthesized in the striatal neurons themselves may more easily stimulate both synaptic and extrasynaptic receptors.

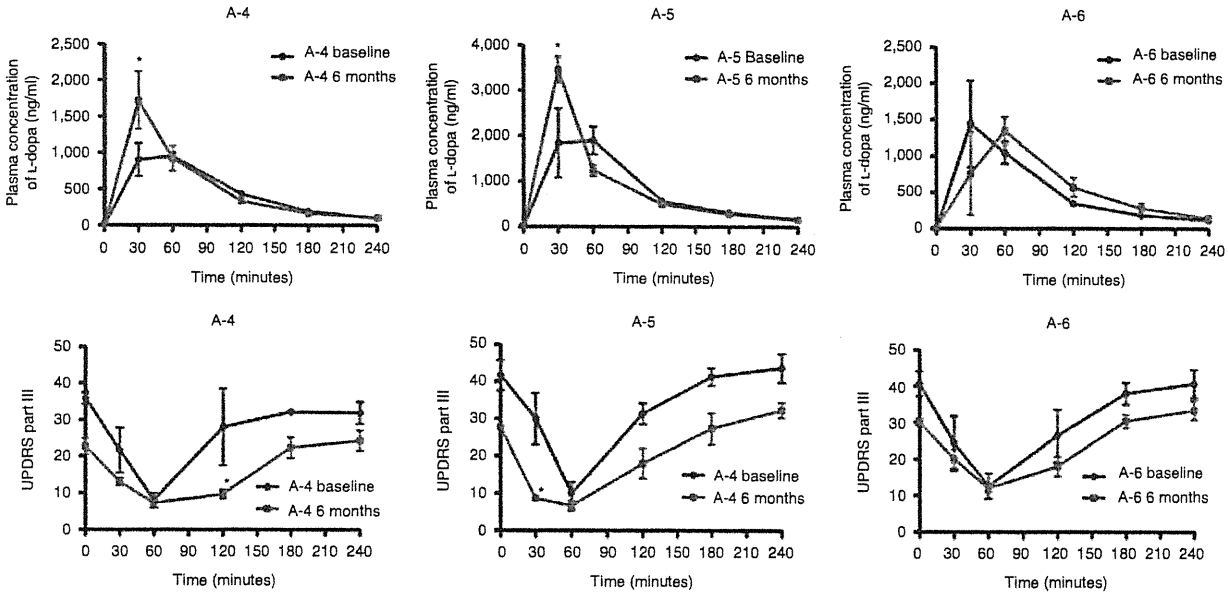

Figure 3 Short-duration response to levodopa. Comparison of short-duration response to levodopa before (blue) and after gene therapy (brown) in three patients (A-4, A-5, and A-6). Patients took 100 mg of levodopa with 25 mg benserazide orally after 20 hours without dopaminergic medication. Values represent means and SE of three trials. Upper panels: plasma levodopa levels; lower panels: Unified Parkinson's Disease Rating Scale motor scores. $^*P < 0.05$.

Results of a similar phase I protocol were reported recently for the 10 patients treated with AAV-hAADC-2 (ref. 10). That study used the same vector preparations as this study. The subjects were divided into two groups that received the same or one-third dose of the vector used in this study, respectively. Although the present patients had slightly milder initial symptoms, the patients treated with the same dose of vector in the two studies showed similar improvement in the OFF state and putaminal FMT uptake on PET. These findings provide independent confirmation of the safety, tolerability, and potential efficacy of AADC gene therapy. Future studies focusing on optimal vector dosing and defining the relationship between vector dose and clinical effects are necessary.[21]

In conclusion, these data indicate that AAV vector–mediated gene transfer of AADC is safe and may benefit advanced PD patients.

MATERIALS AND METHODS

Study design. The protocol and consent forms were approved by the institutional review board. The protocol was also reviewed by the committee of the Ministry of Health, Labour and Welfare of Japan. A data safety monitoring board reviewed the ongoing study. All subjects reviewed the consent form and provided their written, informed consent.

This 24-week, phase I, open-label study was primarily designed to evaluate the safety and tolerability of intraputaminal AAV-hAADC-2 infusion in idiopathic PD. Patients were evaluated preoperatively and monthly postoperatively for 6 months, using multiple measures, including the UPDRS, motor state diaries, the MMSE, the short form of the GDS, and laboratory tests. The UPDRS was done in the practically defined OFF state 12 hours after withdrawal of all antiparkinsonian medications, and in the ON state 1 hour after administration of the usual morning dose of medication. Motor scores for the UPDRS can range from 0 to 56, with higher scores indicating poorer function. Using diaries that separated the day into half-hour segments, the patients recorded their mobility during the 4 days before admission and for another 4 days at 6 months after admission. They were trained to rate their condition as sleeping, immobile, mobile without troublesome dyskinesias, or mobile with troublesome dyskinesias. The total number of hours spent in each of these categories was calculated, and the differences between the baseline and the 6-month scores were compared between the groups.

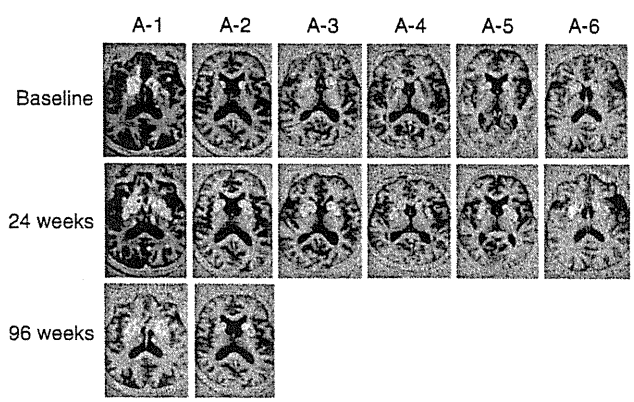

Figure 4 FMT-PET images. Axial images at the level of the putamen are shown before and 24 weeks after gene therapy for all six patients. Increased FMT uptake persisted until 96 weeks in two patients. The 4-week images are not shown because they are similar to the 24-week images. FMT, 6-[^{18}F]fluoro-L-*m*-tyrosine; PET, positron emission tomography.

The short-duration response to levodopa was evaluated in three patients (patients 4–6) at baseline and 6 months after gene transfer; they took 100 mg of levodopa orally with 25 mg benserazide after 20 hours without dopaminergic medication. Motor symptoms based on UPDRS motor (part III) and plasma levodopa concentrations were assessed at baseline and 30 minutes, 1, 2, 3, and 4 hours after levodopa intake.

Patients. The main entry criteria were: age 45–75 years; diagnosis of moderate to advanced PD, defined as Hoehn and Yahr Stage IV and UPDRS in the practically defined OFF condition of at least 20; at least

5 years of levodopa therapy; a minimum 8-point improvement in the UPDRS motor score after levodopa intake; and motor complications not satisfactorily controlled with medical therapy. The main exclusion criteria were atypical parkinsonism, dementia (MMSE score <20), and previous neurosurgical treatment for PD.

Vector and stereotaxic infusion. The vector used in this trial was a recombinant AAV2 with an expression cassette consisting of a human cytomegalovirus immediate-early promoter, followed by the human growth hormone first intron, complementary DNA of human AADC, and simian virus 40 polyadenylation signal sequence.[3–6] Clinical grade AAV-hAADC-2 was manufactured by Avigen (Alameda, CA) and provided by Genzyme (Boston, MA). The patients received AAV-hAADC-2 *via* bilateral intraputaminal infusions. Two target points were determined in the putamen that were sufficiently separated from each other in dorsolateral directions and identified on a magnetic resonance image. One burr hole was trepanned in each side of the cranial bone, through which the vector was injected into the two target points *via* the two-track insertion route. The vector-containing solution was prepared to a concentration of 1.5×10^{12} vector genome/ml, and 50 µl per point of the solution were injected at 1 µl/min; each patient received 3×10^{11} vector genome of AAV-hAADC-2.

Neutralizing antibody titers against AAV2 were determined by measuring β-galactosidase activities in HEK293 cells transduced with 5×10^3 vector genome/cell of AAV2 vectors expressing β-galactosidase in various dilutions of sera.[22]

PET. The AADC expression level in the putamen was assessed on PET imaging with FMT 6 days before surgery and 1 and 6 months after gene transfer. All patients stopped dopaminergic medications 18 hours before PET and took 2.5 mg/kg of carbidopa orally 1 hour before FMT injection. Subsequently, 0.12 mCi/kg of FMT in saline were infused into an antecubital vein, and a 90-minute dynamic acquisition sequence was obtained. The PET and magnetic resonance imaging data were co-registered with a fusion processing program (Syntegra; Philips, Amsterdam, The Netherlands) to produce the fusion images. Radioactivities within volumes of interest drawn in the putamen and occipital lobe were calculated between 80 and 90 minutes after tracer injection. A change in putaminal FMT uptake from baseline to 24 weeks was assessed using the putaminal–occipital ratio of radioactivities.

Statistical analysis. Values at baseline and 6 months after gene transfer were compared using Student's *t*-test (paired analyses). A two-sided *P* value <0.05 was taken to indicate significant differences. Two-way analysis of variance with Bonferroni correction of *P* values was used for the short-duration response to levodopa.

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Characterization of a Recombinant Adeno-Associated Virus Type 2 Reference Standard Material

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Abstract

A recombinant adeno-associated virus serotype 2 Reference Standard Material (rAAV2 RSM) has been produced and characterized with the purpose of providing a reference standard for particle titer, vector genome titer, and infectious titer for AAV2 gene transfer vectors. Production and purification of the reference material were carried out by helper virus–free transient transfection and chromatographic purification. The purified bulk material was vialed, confirmed negative for microbial contamination, and then distributed for characterization along with standard assay protocols and assay reagents to 16 laboratories worldwide. Using statistical transformation and modeling of the raw data, mean titers and confidence intervals were determined for capsid particles ({X}, 9.18×10^{11} particles/ml; 95% confidence interval [CI], 7.89×10^{11} to 1.05×10^{12} particles/ml), vector genomes ({X}, 3.28×10^{10} vector genomes/ml; 95% CI, 2.70×10^{10} to 4.75×10^{10} vector genomes/ml), transducing units

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({X}, 5.09×10^8 transducing units/ml; 95% CI, 2.00×10^8 to 9.60×10^8 transducing units/ml), and infectious units ({X}, 4.37×10^9 $TCID_{50}$ IU/ml; 95% CI, 2.06×10^9 to 9.26×10^9 $TCID_{50}$ IU/ml). Further analysis confirmed the identity of the reference material as AAV2 and the purity relative to nonvector proteins as greater than 94%. One obvious trend in the quantitative data was the degree of variation between institutions for each assay despite the relatively tight correlation of assay results within an institution. This relatively poor degree of interlaboratory precision and accuracy was apparent even though attempts were made to standardize the assays by providing detailed protocols and common reagents. This is the first time that such variation between laboratories has been thoroughly documented and the findings emphasize the need in the field for universal reference standards. The rAAV2 RSM has been deposited with the American Type Culture Collection and is available to the scientific community to calibrate laboratory-specific internal titer standards. Anticipated uses of the rAAV2 RSM are discussed.

Introduction

Recombinant adeno-associated viral (rAAV) vectors are rapidly becoming the gene delivery vehicle of choice for gene transfer, with numerous publications describing their use in animal models and more recently in clinical trials (Warrington and Herzog, 2006; Mueller and Flotte, 2008). The movement of the gene therapy field toward the use of rAAV vectors in clinical trials is largely due to the demonstration of long-term transgene expression in animal models with little associated toxicity and good overall safety profiles in humans (Snyder and Flotte, 2002; Moss *et al.*, 2004; Warrington and Herzog, 2006; Maguire *et al.*, 2008; Mueller and Flotte, 2008; Brantly *et al.*, 2009). Most of the historic data involve rAAV serotype 2 vectors, but vector systems based on other AAV serotypes with more efficient gene delivery profiles in specific tissues are currently in human trials (Brantly *et al.*, 2009; Nienhuis, 2009) and their use will likely increase.

A major problem associated with the body of data to date has been the inability to normalize vector doses administered by different investigators to animals and humans. Thus, there is a need for a reference standard that is recognized by the rAAV research community and that is used to normalize laboratory-specific internal reference standards and test vector titers related to common reference standard units. This need is not new to the field of gene therapy and has previously been addressed for adenoviral vectors. The Adenovirus Reference Material Working Group (ARMWG) developed and characterized the adenovirus reference material (ARM) for the purpose of normalizing titers and doses of gene therapy vectors based on adenovirus type 5 (Hutchins, 2002). Following this example, the U.S. Food and Drug Administration (FDA) and the National Institutes of Health (NIH) Recombinant DNA Advisory Committee (RAC), together with the support of the National Gene Vector Laboratory (NGVL), a program of the NIH National Center for Research Resources (NCRR), encouraged academic and industry scientists within the AAV community to form an AAV Reference Standard Working Group (AAVRSWG) charged with the development a high-quality rAAV reference standard material (Snyder and Flotte, 2002). The AAVRSWG is a volunteer organization and comprises members from both industry and universities in nine different countries, and the International Society for BioProcess Technology (www.ISBioTech.org), under the guidance of the FDA and NIH (Moullier and Snyder, 2008; Potter *et al.*, 2008). Although new serotypes of AAV are currently emerging as efficient gene delivery vectors, the AAVRSWG decided that the first AAV reference standard material should be based on the prototypical AAV serotype 2 because this is by far the best characterized serotype. The approach used in the development of this reference standard lays the groundwork for the development of reference standard materials based on other serotypes. Indeed, a second AAVRSWG has been formed for the development of an AAV8 reference material (Moullier and Snyder, 2008).

The AAV2RSWG recognized that the rAAV2 reference standard material (rAAV2 RSM) must be supplied in sufficient quantity to each requestor for use in all necessary tests at each location, be of high quality, and remain stable for an extended period of time. The group drew up guidelines for the production and purification of the rAAV2 RSM, which were carried out at the Vector Core of the University of Florida's Powell Gene Therapy Center (Gainesville, FL) (Potter *et al.*, 2008). The production process involved cotransfection of batches of ten 10-layer Cell Factories containing HEK293 cells with an AAV2 genome/eGFP transgene plasmid and a second plasmid encoding the AAV2 capsid proteins and necessary helper functions. The transfected cells were harvested and the vector was purified by sequential rounds of column chromatography. A Quality Control subcommittee of the AAV2RSWG was formed for the purpose of characterizing the rAAV2 RSM. In consultation with members of the AAV2RSWG, the committee selected the following characterization assays: (1) capsid titer by A20 enzyme-linked immunosorbent assay (ELISA; Progen Biotechnik, Heidelberg, Germany); (2) vector genome titer by quantitative polymerase chain reaction (qPCR); (3) infectious titer by median tissue culture infective dose ($TCID_{50}$) with qPCR readout and by transduction (green fluorescent protein [GFP] readout); and (4) purity and capsid identification by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

The AAV2 RSM was distributed worldwide to 16 laboratories that volunteered to conduct one or more of the characterization assays. Testing proceeded from July 2008 to March 2009, at which point the quantitative data were collated and statistically analyzed to determine mean titer and confidence intervals. Preliminary analysis showed significant variance and nonnormal data distribution with all of the assays except for particle titer determination. The variance was observed despite providing a standardized protocol and reagents to the testing group and highlights the need within the AAV community for a reference standard with which